Final Report

Original 2 of 2

Determination of the mutagenic potential of [redacted]

with the Bacterial Reverse Mutation Test
following OECD 471 and EU B.13/14

Study No. [redacted]

Sponsor:
[redacted]

Test Facility:
[redacted]

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1 GLP-COMPLIANCE STATEMENT

It is hereby declared that all tests were made in accordance with the „Revised OECD Principles of Good Laboratory Practice" (Paris, 1997) as stated in the following guidelines:

- OECD Principles of Good Laboratory Practice, adopted by Council on 26th November 1997; Environment Directorate, Organisation for Economic Cooperation and Development, Paris 1998
- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version)
- Chemikaliengesetz (Chemicals Act) of the Federal Republic of Germany (ChemG) §19a and §19b and annexes 1 and 2 in the version of 02 July 2008 published in Bundesgesetzblatt No. 28/2008, pp. 1146 - 1184

Responsibility for the accuracy of the information concerning the test item as well as for its authenticity rests with the sponsor.

I herewith accept responsibility for the data presented within this report.

There were no circumstances that may have affected the quality or integrity of the study.

[redacted]

[redacted]

Information on Study Organisation:

Deputy Study Director [redacted]

Study Plan dated

Experimental Starting Date

Experimental Completion Date

Draft Report dated

2 Quality Assurance Unit Statement

This study has been inspected by the quality assurance unit according to the principles of Good Laboratory Practice. Study Plan and Final Report were checked at the dates given below, the Study Director and the management were informed with the corresponding report.

Also, the performance of the study was inspected, and findings were reported to Study Director and management. The inspection of short-term studies (duration less than four weeks) is carried out as audit of process concerning major technical phases of at least one similar test. Frequency is once or more a quarter.

The study was conducted and the reports were written in accordance with the Study Plan and the Standard Operating Procedures of the test facility.

Deviations from the Study Plan were acknowledged and assessed by the Study Director and included in the Final Report.

The reported results reflect the raw data of the study.

Verified Procedure	Inspected on	Findings reported on	Audit report no.
Study plan	[redacted]		
Performance of study			
Draft report			
Final report			

[redacted]

[redacted]

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3 SUMMARY

Title of Study: Determination of the mutagenic potential o[redacted]with the Bacterial Reverse Mutation Test following OECD 471 and EU B.13/14

Findings and Results:

Two valid experiments were performed. One invalid experiment was performed, only the toxicity control was valid and was evaluated.

First Experiment:

Five concentrations of the test item, suspended in deionised water (ranging from 4995 to 51 µg/plate) were used. Five genetically manipulated strains of *Salmonella typhimurium* (TA 97a, TA 98, TA 100, TA 102 and TA 1535) were exposed to the test item both in the presence and in the absence of a metabolic activation system (S9) for 48 hours, using the plate incorporation method.

None of the concentrations caused a significant increase in the number of revertant colonies in the tested strains. The test item didn't show any mutagenic effects in the first experiment.

No signs of toxicity towards the bacteria could be observed.

The sterility control and the determination of the titre didn't show any inconsistencies. The determined values for the spontaneous revertants of the negative controls were in the normal range. All positive controls showed mutagenic effects with and without metabolic activation.

Second Experiment:

To verify the results of the first experiment, a second experiment was performed, using five concentrations of the test item (ranging from 4999 to 312 µg/plate) and a modification in study performance (pre-incubation method).

The test item didn't show mutagenic effects in the second experiment, either.

No signs of toxicity towards the bacteria could be observed.

The sterility control and the determination of the titre didn't show any inconsistencies. The determined values for the spontaneous revertants of the negative controls were in the normal range. All positive controls showed mutagenic effects with and without metabolic activation.

Under the conditions of the test, the test item didn't show mutagenic effects towards *Salmonella typhimurium,* strains TA 97a, TA 98, TA 100, TA 102 and TA 1535.

Therefore, no concentration-effect relationship could be determined.

The test item[redacted]s considered as
"not mutagenic under the conditions of the test".

4 PURPOSE AND PRINCIPLE OF THE STUDY

The bacterial reverse mutation test uses amino-acid requiring strains of Salmonella typhimurium to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs. The principle of this bacterial reverse mutation test is that it detects mutations which revert mutations present in the test strains and restore the functional capability of the bacteria to synthesize an essential amino acid.

The bacterial reverse mutation test is commonly employed as an initial screen for genotoxic activity and, in particular, for point mutation-inducing activity.

Suspensions of bacterial cells are exposed to the test substance in the presence and in the absence of an exogenous metabolic activation system. In the plate incorporation method, these suspensions are mixed with an overlay agar and plated immediately onto minimal medium. In the preincubation method, the treatment mixture is incubated and then mixed with an overlay agar before plating onto minimal medium. For both techniques, after two or three days of incubation, revertant colonies are counted and compared to the number of spontaneous revertant colonies on solvent control plates.

This study was performed in order to evaluate the mutagenic potential of [redacted] in the Bacterial Reverse Mutation Test. The test was chosen on behalf of the sponsor.

Sponsor's intent: REACH.

5 LITERATURE

The study was conducted in accordance with the following guidelines:

- OECD Guidelines for the Testing of Chemicals Part 471, adopted 21. Jul. 1997 "Bacterial Reverse Mutation Test"
- EU-Guideline B.13/14 adopted 31. May 2008 "Mutagenicity –Reverse mutation test using bacteria"

Corresponding SOP of [redacted]

- [redacted]

6 MATERIALS AND METHODS

6.1 Test Item

6.1.1 Specification

The following information concerning identity and composition of the test item were provided by the sponsor.

Name	[redacted]
Batch no	
Appearance	
Composition	
CAS-No.	
EINECS-No.	
Molecular formula	
Molecular weight	
Purity	
Homogeneity	
Volatility	
Stability	
Solubility	
Date of production	
Date of expiry	
Storage conditions	
Hazard information	
R-phrases	
S-phrases	

6.1.2 Storage

The test item was stored in the test facility at room temperature.

6.1.3 Preparation

The test item was not completely soluble in the solvents for the AMES test. On demand of the sponsor, sterile H_2O demin. was used as medium for a suspension.

Therefore, the test item was weighed directly for all tested concentrations and was autoclaved and suspended in sterile H_2O demin. The suspension was stirred for 24 hours before the start of each experiment as well as during the test.

The following (weighed) concentrations were used in the first experiment:

4995 / 1527 / 500 / 151 / 51 µg/plate.

The following (weighed)concentrations were used in the second experiment:

4999 / 2528 / 1254 / 628 / 312 mg/plate.

6.2 Positive Controls

The following mutagenic substances were used as positive controls:

6.2.1 4-Nitro-1,2-phenylene diamine

4-Nitro-1,2-phenylene diamine, $C_6H_7N_3O_2$; CAS-No.: 99-56-9

Concentration per plate:	20 µg
Solvent	DMSO
Strains:	TA 97a, TA 98 and TA 102
Metabolic activation:	none

6.2.2 Sodium azide

Sodium azide, NaN_3; CAS-Nr.: 26628-22-8

Concentration per plate:	1 µg
Solvent	H_2O
Strains:	TA 100 and TA 1535
Metabolic activation:	none

6.2.3 2-Amino-anthracene

2-Amino-anthracene, $C_{14}H_{11}N$; CAS-Nr.: 613-13-8

Concentration per plate:	1 µg
Solvent	DMSO
Strain:	TA 97a, TA 100, TA 102 and TA 1535.
Metabolic activation:	S9

6.2.4 Benzo-a-pyrene

Benzo-a-pyrene, $C_{20}H_{12}$; CAS-Nr.: 50-32-8

Concentration per plate:	20 µg
Solvent	DMSO
Strain:	TA 98
Metabolic activation:	S9

6.3 Test System

6.3.1 Specification

Species	*Salmonella typhimurium* LT2
Strains	TA 1535, TA 97a, TA 98, TA 100 and TA 102
Mutations	TA97a: hisD6610, uvrB, pKM 101, rfa
	TA 98: hisD3052, uvrB, pKM 101, rfa
	TA 100: hisG46, uvrB, pKM 101, rfa
	TA102: hisG428, pKM 101, rfa
	TA1535: hisG46, uvrB, rfa.

6.3.2 Origin and Culture

Salmonella typhimurium (all strains used) were obtained from TRINOVA BioChem (date of arrival for strains TA98, TA100 and TA1535: 04. March 2009; date of arrival for strain TA102: 10. Mar. 2008; date of arrival for strain TA97a: 11. Mar. 2010) and were stored as lyophilisate in the fridge at 2-5°C.

The lyophilisates were used to prepare permanent cultures which were filled into vials and stored at < -75°C.

One day before the start of each experiment, one vial per strain to be used was taken from the deep freezer. The surface was scraped with an inoculation loop and the aliquot was put into a culture vessel containing nutrient broth. After incubation over night at 37°C, the cultures were used in the experiment. During the test, the cultures were stored at room temperature as to prevent changes in the titre.

6.4 Chemicals

The purity of the chemicals which were used were either "analytical grade" or "for microbiological purposes".All solutions and media were sterilized, either by autoclaving (121 °C, 20 minutes) or by membrane filtration. The weights may differ from the theoretical value (difference < 10 %). Additionally, differing specifications (e.g. water of crystallization) may be used.

6.4.1 Nutrient broth for overnight culture

Nutrient broth Merck 5443	2.8 g
H_2O demin.	ad 350 mL

6.4.2 Isotonic sodium chloride solution for dilution purposes

Sodium chloride	0.9 g
H_2O demin.	ad 100 mL

6.4.3 Vogel-Bonner-Medium 20fold

Magnesium sulphate ($MgSO_4*7H_2O$)	4.0 g
Citric acid mono hydrate	40.0 g
Potassium phosphate, dibasic (anhydrous) (K_2HPO_4)	200.0 g
Sodium ammonium phosphate, monobasic, tetra hydrate ($Na(NH_4)HPO_4*4H_2O$)	70.0 g
H_2O demin.	ad 1000.0 mL

6.4.4 Glucose solution 40%

Glucose	400.0 g
H_2O demin.	ad 1000.0 mL

6.4.5 Minimal Glucose Agar

Vogel-Bonner-Solution 20fold	500.0 mL
Glucose solution 40%	500.0 mL
H_2O demin.	9000.0 mL
Agar	150.0 g

6.4.6 Biotin Agar

Minimal-Glucose-Agar, 80 °C	500.0 mL
Biotin solution 0.5 millimolar	3.0 mL

6.4.7 Histidine-Biotin-Agar

Biotin-Agar, 80 °C	350.0 mL
Histidine solution 0.5%	3.5 mL

6.4.8 Ampicillin-Agar

Histidine-biotin agar, 80 °C	250.0 mL
Ampicillin solution 0.8%	0.6 mL

6.4.9 Ampicillin-tetracycline plates

Ampicillin agar, 80 °C	50.0 mL
Tetracycline solution 0.8%	0.01 mL

6.4.10 Nutrient agar plates

Nutrient broth Merck 5443	0.8 g
Sodium chloride (NaCl)	0.5 g
Agar	1.52 g
H_2O demin.	100 mL

6.4.11 Basis for Top-Agar and Maximal-Soft-Agar

Agar	6 g
Sodium chloride (NaCl)	5 g
H_2O demin.	ad 1000 mL

6.4.12 Histidine-Biotin-solution 0.5/0.5 millim. (use: top agar)

D-Biotin (MG 247.3)	12.36 mg
L-Histidine* HCl*1H_2O (MG 209.7 g/mol)	10.5 mg
H_2O demin., 90°C	ad 100.0 mL

To 100 mL basis (see 6.4.11), 10 mL histidine-biotin-solution 0.5/0.5 millim. are added.

6.4.13 Histidine-Biotin-solution 5 millim./ 0.5 millim. (use: maximal-soft-agar)

D-Biotin (MG 247.3)	12.36 mg
L-Histidine* HCl*1H_2O (MG 209.7 g/mol)	105 mg
H_2O demin., 90°C	ad 100.0 mL

To 100 mL basis (see 6.4.11),10 mL histidine-biotin-solution 5/0.5 millim. are added.

6.4.14 Phosphate buffer

Sodium di-hydrogen phosphate monohydrate $NaH_2PO_4*H_2O$	0.3312 g
Di-sodium hydrogen phosphate Na_2HPO_4	2.5 g
H_2O demin.	ad 100.0 mL

The pH of the solution is adjusted to 7.4 ± 0.1.

6.4.15 Salt solution for S9-Mix

Potassium chloride (KCl)	1.23 g
Magnesium chloride hexahydrate $MgCl_2*6H_2O$	0.814 g
H_2O demin.	ad 10.0 mL

6.4.16 NADP-solution for S9-Mix

NADP (M_R = 765.4 g/mol)	766 mg
H_2O demin.	10 mL

6.4.17 Glucose-6-phosphate solution for S9-Mix

Glucose-6-phosphate (M_R = 282 g/mol)	564 mg
H_2O demin., sterile	ad 2 mL

6.4.18 S9-Mix

Phosphate buffer	22,5 mL
0.1m NADP-solution	1.0 mL
1m-G6P-solution	0.125 mL
Salt solution	0.5 mL
Rat liver S9, 4%	1.0 mL

6.4.19 S9

S9 was obtained by Trinova Biochem, Gießen.

Batch no:	2672, 2715
Specification:	produced from the livers of male Sprague-Dawley rats which were treated with 500 mg Aroclor 1254/kg body weight intra-peritoneally.

6.5 Test Vessels

All vessels used are made of glass or sterilizable plastic. They were sterilized before use by heating to 180 °C (two hours) or autoclaving.

The following vessels were used:

- Schott-bottles, glass vials, and culture flasks for solutions and media
- Plastic petri plates
- Glass tubes (with Kapsenberg caps) for TopAgar-bacteria-substance mix

6.6 Instruments and Devices

The following instruments and devices were used in the performance of the study.

- Sanyo Labo Autoclave MLS 3020
- Magnetic stirrer #1 and magnetic stir bars
- Precision scales Mettler PB 5001-S02 Labostyle 5001
- Precision scales Mettler XS6001S
- Analytical scales Mettler Toledo XS205DU [redacted]
- Incubation chambers Heraeus, adjustable to 37 °C , [redacted]
- Table water bath GFL, adjustable to 43°C
- Heating chamber Memmert [redacted]
- Orbital shaker GFL 3005 No.:8
- Piston-driven pipettes with sterile tips, [redacted] (0.2 - 2 mL), 22 (20-200 µL), 32-33 (500 µL), 34-38 (100 µL)
- Glass thermometer [redacted]
- Glass measuring cylinders, 100 and 1000 mL
- Glass measuring flasks, 25, 500 and 1000 mL
- Membrane filters, 0.2 µm pore diameter
- Pipetting device Accu Jet
- Tally counters [redacted]
- pH meter wtw 340i

Usage and, if applicable, calibration of all instruments following the corresponding SOP in the current edition.

7 PERFORMANCE OF THE STUDY

7.1 Culture of Bacteria

The lyophilisates of the strains were used to prepare permanent cultures which were filled into vials and stored at < -75 °C.

One day before the start of each experiment, one vial per strain to be used was taken from the freezer and an aliquot was put into a culture vessel containing nutrient broth. After incubation for 8 hours at 37 °C, the cultures were used in the experiment. During the test, the cultures were stored at room temperature as to prevent changes in the titre.

7.2 Conduct of Experiment

7.2.1 Preparations

In the days before each test, the media and solutions were prepared. Two days before the test, the plates were sterilized and the first batches poured. On the day before the test, the remaining plates were poured.

On the day of the test, the overnight cultures were checked for growth. The incubation chambers were heated to 37 °C. The water bath was turned to 43 °C. The table surface was disinfected.

The S9 mix was freshly prepared and stored at 0 °C.

7.2.2 Experimental Parameters

7.2.2.1 First Experiment

Date of treatment	[redacted]
Concentrations tested	4995 / 1527 / 500 / 151 / 51 µg/plate
Incubation time	48 hours
Incubation temperature	37 °C
Tester strains	TA97a, TA98, TA100, TA102, TA1535
Method	plate incorporation method
Positive controls	see chapter 7.3.6

7.2.2.2 Second Experiment

Date of treatment	[redacted]
Concentrations tested	4999 / 2528 / 1254 / 628 / 312 µg/plate
Incubation time	48 hours
Incubation temperature	37 °C
Tester strains	TA97a, TA98, TA100, TA102, TA1535
Method	pre-incubation method
Positive controls	see chapter 7.3.6

7.2.3 Description of the Method

7.2.3.1 General preparation

Per strain and dose, four plates with and four plates without S9 mix were used.

One day before the start of the test, for all concentrations, the test item was weighed directly into sterile test tubes and autoclaved; then, the tubes were filled up with aqua demin. All suspensions were constantly stirred over night and during the test.

Top agar basis was melted in a microwave oven, after melting, 10 mL of histidine-biotin-solution 0.5 mMol per 100 mL basis was added and the bottle was placed in the water bath at 43 °C.

7.2.3.2 Plate incorporation method

The following materials were mixed in a test tube and poured onto the selective agar plates:

- 100 µL test suspension at each dose level, solvent (negative control) or reference mutagen solution (positive control)
- 500 µL S9 mix (see chapter 6.4.18, for test with metabolic activation) or phosphate buffer (for test without metabolic activation).
- 100 µL bacteria suspension (cf. test system, culture of the strains)
- 2000 µL overlay agar (top agar)

The plates were closed and left to harden for a few minutes, then inverted and placed in the dark incubator at 37°C.

The applied volumes correspond to the following test item concentrations:

4995 µg/plate; 1527 µg/plate; 500 µg/plate; 151 µg/plate; 51 µg/plate.

7.2.3.3 Pre-incubation method

The following materials were mixed in a test tube and incubated at 37 °C for 20 minutes:

- 100 µL test suspension at each dose level, solvent (negative control) or reference mutagen solution (positive control)
- 500 µL S9 mix (see chapter 6.4.18, for test with metabolic activation) or phosphate buffer (for test without metabolic activation).
- 100 µL bacteria suspension (cf. test system, culture of the strains)

After pre-incubation, 2000 µL overlay agar (top agar) was added and the mixture was poured onto the selective agar plate.

The plates were closed and left to harden for a few minutes, then inverted and placed in the dark incubator at 37°C.

The applied volumes correspond to the following test item concentrations:

4999 µg/plate; 2528 µg/plate; 1254 µg/plate; 628 µg/plate; 312 µg/plate.

7.3 References and Validity

7.3.1 Genotype Confirmation
Genotype confirmation is performed once a quarter.

7.3.1.1 Histidine requirement
Each strain was streaked on a biotin and a histidine-biotin-plate, using a sterilized wire loop. The plates were incubated for 24 hours.

7.3.1.2 Ampicillin-Resistance (pKM 101) resp. ampicillin-tetracycline-resistance (pAQ1)
The strains were streaked on ampicillin agar, TA102 on ampicillin-tetracycline agar. TA1535 was taking the function of control strain, since it is not ampicillin resistant. The plates were incubated for 24 hours at 37°C.

7.3.1.3 UV-sensitivity (uvrB)
Two plates were streaked with the five strains, and one half of the plate covered with aluminium foil so that one half of each streak was protected against light. The plates for the strain TA97a, TA98, TA100 and TA102 were irradiated for 8 seconds, the plates for the strain TA1535 were irradiated for 6 seconds with a germicidal lamp (254 nm, 30W), keeping a distance of 33 cm. Incubation over night at 37°C followed.

7.3.1.4 Crystal violet sensitivity (deep rough)
For each strain, two plates were used. 0.1 mL of bacteria suspension were mixed with 2 mL Top-Agar and poured on nutrient agar. Sterile paper discs (∅ 9 mm), each soaked with 10 µl of crystal violet solution (0.1%) were placed into the middle of each plate, followed by incubation over night.

7.3.2 Spontaneous Revertants
Four replicates, with/without S9, for each solvent which was used in the test.

7.3.3 Determination of Titre
The titre was determined by dilution of the overnight culture using sodium chloride solution and placing 0.1 mL on maximal-soft agar. It should give a density of 10^9 cells/mL (at the least).

7.3.4 Toxicity Control
Performed in experiment 1 only analogously to the titre control with the maximum dose of test item with and without S9 on maximal-soft agar.

7.3.5 Sterility Control
Performed analogously to the test with solvent only and S9 (without adding bacteria) on top agar.

7.3.6 Positive Controls
Using diagnostic mutagens (see chapter 6.2), four replicates were prepared. The stock solutions of the substances were diluted to effect an application volume of 0.1 mL/plate.

7.4 Evaluation

The colonies were counted visually, the numbers were recorded. A spreadsheet software (Microsoft Excel®) was used to calculate mean values and standard deviations of each treatment, solvent control and positive control.

The increase factor f(I) of revertant induction (mean revertants divided by mean spontaneous revertants) and the absolute number of revertants ("Rev. abs.", mean revertants less mean spontaneous revertants) were calculated, too.

A test item is considered to have mutagenic potential, if a significant, reproducible increase of revertant colonies per plate (increase factor ≥ 2) in at least one strain can be observed. A concentration-related increase over the range tested can also be taken as a sign of mutagenic activity.

8 FINDINGS

The detailed data of the two experiments are listed in the annex (Exp. 1 see page 27 ff., Exp. 2 see page 32).

Confirmation of genotype is performed once a quarter. The last performance showed no abnormalities (see page 39).

8.1 First Experiment

8.1.1 Confirmation of the Criteria and Validity

The treatments for the sterility control and the determination of the titre didn't show any inconsistencies. The determined values for the spontaneous revertants of the negative controls were in the normal range of the test laboratory (historical data of the laboratory see page 38). All positive controls (diagnostic mutagenes) showed mutagenic effects with and without metabolic activation.

8.1.2 Solubility and Toxicity

The test item was suspended in deionised water. All concentrations were weighed directly.

No signs of toxicity towards the tested strains could be observed. The background lawn was visible and the number of revertant colonies was not reduced.

8.1.3 Mutagenicity

No significant increase of the number of revertant colonies in the treatments with and without metabolic activation could be observed. No concentration-related increase over the tested range was found.

Therefore the test item is stated as not mutagenic under the test conditions.

To verify this result, a second experiment was performed using the pre-incubation method.

8.1.4 Survey of the Findings

The mean revertant values of the four replicates are presented in the following table.

Table 8.1-a Mean Revertants First Experiment

Strain		TA97a		TA98		TA100		TA102		TA1535	
Induction		-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
H_2O	Mean	124	113	15	18	107	103	166	194	16	18
	sd	5.8	6.5	2.1	2.5	8.1	7.0	6.8	15.6	3.2	2.6
DMSO	Mean	117	116	19	21	109	112	195	185	21	14
	sd	8.7	6.5	1.8	4.2	3.5	2.6	12.7	28.4	2.6	3.0
Pos.Contr.	Mean	618	719	302	370	669	686	591	472	150	121
	sd	129	31	59	10	59	25	45	115	15	24
	f(I)	5.28	6.20	15.89	17.62	6.25	6.13	3.03	2.55	9.38	8.64
4999 µg/pl.	Mean	112	119	12	12	116	108	154	213	15	16
	sd	5	4	3	2	6	15	11	10	3	3
	f(I)	0.90	1.05	0.80	0.67	1.08	1.05	0.93	1.10	0.94	0.89
1527 µg/pl.	Mean	117	118	15	9	110	122	216	189	13	14
	sd	4	2	1	2	5	15	5	9	4	3
	f(I)	0.94	1.04	1.00	0.50	1.03	1.18	1.30	0.97	0.81	0.78
500 µg/pl.	Mean	119	117	15	13	120	115	211	182	10	16
	sd	8	5	5	1	8	23	19	13	1	2
	f(I)	0.96	1.04	1.00	0.72	1.12	1.12	1.27	0.94	0.63	0.89
151 µg/pl.	Mean	109	120	18	12	116	127	198	220	13	15
	sd	6	6	3	2	3	16	22	4	4	1
	f(I)	0.88	1.06	1.20	0.67	1.08	1.23	1.19	1.13	0.81	0.83
50 µg/pl.	Mean	119	119	9	15	114	109	216	210	12	17
	sd	7	2	2	4	2	6	24	13	4	2
	f(I)	0.96	1.05	0.60	0.83	1.07	1.06	1.30	1.08	0.75	0.94

f(I) = increase factor, calculation see chapter „Evaluation“, page 19

8.2 Second Experiment

8.2.1 Confirmation of the Criteria and Validity

The treatments for the sterility control and the determination of the titre didn't show any inconsistencies. The determined values for the spontaneous revertants of the negative controls were in the normal range of the test laboratory. All positive controls showed mutagenic effects with and without metabolic activation.

8.2.2 Solubility and Toxicity

The test item was suspended in deionised water. All concentrations were weighed directly.

No signs of toxicity towards the tested strains could be observed. The background lawn was visible and the number of revertant colonies was not significantly reduced.

8.2.3 Mutagenicity

No significant increase of the number of revertant colonies in the treatments with and without metabolic activation was observed. No concentration-related increase over the tested range was found.

Therefore the test item is stated as not mutagenic under the test conditions.

8.2.4 Survey of the Findings

The mean revertant values of the four replicates are presented in the following table.

Table 8.2-a Mean Revertants Second Experiment

Strain		TA97a		TA98		TA100		TA102		TA1535	
Induction		-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
H_2O	Mean	115	124	10	12	110	122	214	223	18	14
	sd	5.4	8.9	0.0	1.3	18.9	16.8	15.7	10.1	4.6	2.2
DMSO	Mean	111	136	15	18	124	124	220	233	15	17
	sd	4.3	18.1	4.6	5.0	32.8	23.5	8.9	17.3	0.5	3.0
Pos.Contr.	Mean	697	522	170	126	430	575	595	501	228	106
	sd	165	48	38	11	81	128	76	30	25	5
	f(I)	6.28	3.84	11.33	7.00	3.91	4.64	2.70	2.15	12.67	6.24
4999 µg/pl.	Mean	122	115	13	13	123	105	225	211	17	16
	sd	8	7	3	3	5	1	29	32	6	1
	f(I)	1.06	0.93	1.30	1.08	1.12	0.86	1.05	0.95	0.94	1.14
2528 µg/pl.	Mean	110	116	11	10	122	101	234	228	15	18
	sd	5	9	3	1	5	1	17	15	4	3
	f(I)	0.96	0.94	1.10	0.83	1.11	0.83	1.09	1.02	0.83	1.29
1254 µg/pl.	Mean	118	112	15	12	120	109	218	228	18	18
	sd	10	5	1	1	2	5	23	26	3	1
	f(I)	1.03	0.90	1.50	1.00	1.09	0.89	1.02	1.02	1.00	1.29
628 µg/pl.	Mean	113	112	13	14	123	107	229	223	16	17
	sd	7	5	2	3	2	2	24	9	5	2
	f(I)	0.98	0.90	1.30	1.17	1.12	0.88	1.07	1.00	0.89	1.21
312 µg/pl.	Mean	113	116	10	15	126	111	236	229	19	16
	sd	6	12	0	1	5	5	9	12	2	5
	f(I)	0.98	0.94	1.00	1.25	1.15	0.91	1.10	1.03	1.06	1.14

f(I) = increase factor, calculation see chapter „Evaluation", page 19

9 RESULTS

9.1 Mutagenicity of Test Item

The test item didn't show mutagenic effects in both experiments. The number of revertant colonies was not increased in comparison with the spontaneous revertants (solvent only).

Cytotoxicity of the test item was not detected. The background lawn was visible and the number of revertants was not significantly decreased.

Therefore it can be stated, that under the test conditions, the test item [redacted] is not mutagenic in the Bacterial Reverse Mutation Test using *Salmonella typhimurium,* strains TA 97a, TA 98, TA 100, TA 102 and TA 1535.

9.2 Acceptability of Study

Nearly all spontaneous revertants and all positive control values were within the range of the historical data. Difference of revertants lying outside the range are marginal. Therefore, the study is considered valid.

10 DISCUSSION

The test item is considered not mutagenic for the reasons given above.

Also, the test item didn't show any cytotoxicity towards the bacteria.

Two valid experiments were performed. The first experiment was invalid; only the toxicity control was valid and could be evaluated. The raw data of the invalid experiment will be archived with the raw data of the valid experiments.

The confirmation tests of the genotype didn't show any irregularities. The control of the titre was above the demanded value. The number of revertant colonies of the positive controls were in the range of the historical data of the laboratory (see page 38) and were definitely increased in comparison with the negative controls, as well as showing mutagenous potential of the diagnostic mutagenes.

Spontaneous revertants were within the normal range in comparison with the historical data of the [redacted]

For these reasons, the result of the test is considered valid.

11 DEVIATIONS

11.1 Deviations from the Study Plan

No deviations from the study plan were observed.

11.2 Deviations from the Guideline

None as known.

12 RECORDING

One original of study plan and final report, respectively, all raw data of the study and all documents mentioned or referred to in study plan or final report will be kept in the GLP Document Archive of the test facility for fifteen years. After that, the sponsor's instructions will be applied (shipment of documentation to sponsor). A retain sample of the test item will be kept in the GLP Substance Archive for fifteen years; then, the retain sample will be discarded.
Number of originals which will be sent to the sponsor: 1

13 ANNEX 1: COPY OF GLP CERTIFICATE

RheinlandPfalz
LANDESAMT FÜR UMWELT, WASSERWIRTSCHAFT UND GEWERBEAUFSICHT

GUTE LABORPRAXIS – GOOD LABORATORY PRACTICE

GLP-BESCHEINIGUNG
STATEMENT OF GLP COMPLIANCE

gemäß/according to § 19b Abs. 1 Chemikaliengesetz

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EC at:

Prüfeinrichtung / Test facility

[redacted]

Prüfung nach Kategorien / Areas of Expertise
(gemäß / according ChemVwV-GLP Nr. 5.3/OECD guidance)
1, 3, 4, 5, 6 , 8, 9 (toxikologische in Vitro Prüfungen an Säugerzellen und Bakterien)

Datum der Inspektion / Date of inspection
(Tag.Monat.Jahr / day.month.year)
29. und 30. November 2010

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that the test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Eine erneute behördliche Überprüfung der Einhaltung der GLP-Grundsätze durch die Prüfeinrichtung ist so rechtzeitig zu beantragen, dass die Folgeinspektion spätestens vier Jahre nach dem Beginn der o.g. Inspektion stattfinden kann. Ohne diesen Antrag wird die Prüfeinrichtung nach Ablauf der Frist aus dem deutschen GLP-Überwachungsprogramm genommen und diese GLP-Bescheinigung verliert ihre Gültigkeit.

Verification of the compliance of the test facility with the Principles of the GLP has to be applied for in time to allow for a follow-up inspection to take place within four years after commencing the above mentioned inspection. Elapsing this term, the test facility will be taken out of the German GLP-Monitoring Programme and this GLP Certificate becomes invalid.

Unterschrift, Datum / Signature, Date

[signature] 24.08.11

Dr.-Ing. Stefan Hill - Präsident -
(Name und Funktion der verantwortlichen Person / name and function of responsible person)

Landesamt für Umwelt, Wasserwirtschaft und Gewerbeaufsicht
Kaiser-Friedrich-Straße 7, 55116 Mainz
(Name und Adresse der GLP-Überwachungsbehörde / Name and adress of the GLP Monitoring Authority)

MESSEN BEWERTEN BERATEN

14 ANNEX 2: DATA OF THE FIRST EXPERIMENT

14.1 Determination of Titre

Criterion: The determination of titre should give a number of at least 10^9 cells/mL, correlating to 100 colonies/plate after dilution.

Table 14.1-a Titre Values (colonies per plate)

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
Repl. 1	376	314	310	342	338	380	340	380	230	300
Repl. 2	380	332	320	314	362	420	360	362	246	282
Mean	**378**	**323**	**315**	**328**	**350**	**400**	**350**	**371**	**238**	**291**
sd	**3**	**13**	**7**	**20**	**17**	**28**	**14**	**13**	**11.3**	**12.7**
Assessment	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok

14.2 Sterility Control

Criterion: At the maximum, one colony per plate may grow.

Table 14.2-a Sterility (colonies per plate)

	H_2O	DMSO
Repl. 1	0	0
Repl. 2	0	0
Repl. 3	0	0
Repl. 4	0	0
Assessment	**ok**	**ok**

14.3 Spontaneous Revertants

The determined values were within the normal range of the laboratory.

14.3.1 Water

Table 14.3-a Spontaneous Revertants H_2O (colonies per plate)

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Repl. 1	125	105	13	15	95	108	166	212	13	17
Repl. 2	120	113	15	17	113	100	170	182	17	19
Repl. 3	118	113	18	17	111	110	156	202	14	15
Repl. 4	131	121	15	21	108	95	171	180	20	21
Mean	**124**	**113**	**15**	**18**	**107**	**103**	**166**	**194**	**16**	**18**
sd	**5.8**	**6.5**	**2.1**	**2.5**	**8.1**	**7.0**	**6.8**	**15.6**	**3.2**	**2.6**

14.3.2 DMSO

Table 14.3-b Spontaneous Revertants DMSO (colonies per plate)

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Repl. 1	128	120	18	25	113	114	212	206	20	10
Repl. 2	113	117	21	22	110	110	182	162	22	17
Repl. 3	120	106	20	15	107	110	194	158	23	15
Repl. 4	108	119	17	20	105	115	190	212	17	15
Mean	**117**	**116**	**19**	**21**	**109**	**112**	**195**	**185**	**21**	**14**
sd	**8.7**	**6.5**	**1.8**	**4.2**	**3.5**	**2.6**	**12.7**	**28.4**	**2.6**	**3.0**

14.4 Positive Controls

- Without metabolic activation:
  4-Nitro-1,2-phenylene diamine (NPD) in DMSO, 20 µg/plate
  Sodium azide (Na-azide) in deionised water, 1 µg/plate
- With metabolic activation:
  2-Amino anthracene (2-AA) in DMSO, 1 µg/plate
  Benzo-a-pyrene (BaP) in DMSO, 20 µg/plate

Table 14.4-a Diagnostic Mutagenes (colonies per plate)

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Substance	NPD	2-AA	NPD	BaP	Na-azide	2-AA	NPD	2-AA	Na-azide	2-AA
Repl. 1	792	760	236	380	724	680	532	372	133	133
Repl. 2	480	712	302	374	712	660	604	380	151	105
Repl. 3	608	720	290	370	640	720	640	604	170	97
Repl. 4	592	684	380	356	600	684	588	532	147	149
Mean	**618**	**719**	**302**	**370**	**669**	**686**	**591**	**472**	**150**	**121**
sd	**129**	**31**	**59**	**10**	**59**	**25**	**45**	**115**	**15**	**24**
f(I)	**5.28**	**6.20**	**15.89**	**17.62**	**6.25**	**6.13**	**3.03**	**2.55**	**9.38**	**8.64**
Rev. abs.	**501**	**603**	**283**	**349**	**562**	**574**	**396**	**287**	**134**	**107**

f(I) = increase factor, calculation see chapter „Evaluation", page 19
Rev.abs. = absolute revertants, calculation see chapter „Evaluation", page 19

14.5 Test Item [redacted]

14.5.1 Mutagenicity Test

Table 14.5-a Concentration 4995 µg/plate (colonies per plate)

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
Repl. 1	119	120	10	13	121	96	145	210	14	14
Repl. 2	110	122	17	11	118	116	151	202	11	13
Repl. 3	107	120	10	13	108	124	150	214	17	17
Repl. 4	113	113	12	9	115	94	171	226	17	20
Mean	**112**	**119**	**12**	**12**	**116**	**108**	**154**	**213**	**15**	**16**
sd	**5**	**4**	**3**	**2**	**6**	**15**	**11**	**10**	**3**	**3**
f(l) .	**0.90**	**1.05**	**0.80**	**0.67**	**1.08**	**1.05**	**0.93**	**1.10**	**0.94**	**0.89**
Rev. abs.	**-12**	**6**	**-3**	**-6**	**9**	**5**	**-12**	**19**	**-1**	**-2**

Table 14.5-b Concentration 1527 µg/plate (colonies per plate)

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
Repl. 1	113	120	16	12	113	112	211	200	13	13
Repl. 2	115	117	16	9	103	128	214	191	13	9
Repl. 3	121	115	14	9	109	141	216	178	9	17
Repl. 4	120	118	14	6	115	108	222	185	18	15
Mean	**117**	**118**	**15**	**9**	**110**	**122**	**216**	**189**	**13**	**14**
sd	**4**	**2**	**1**	**2**	**5**	**15**	**5**	**9**	**4**	**3**
f(l)	**0.94**	**1.04**	**1.00**	**0.50**	**1.03**	**1.18**	**1.30**	**0.97**	**0.81**	**0.78**
Rev. abs.	**-7**	**5**	**0**	**-9**	**3**	**19**	**50**	**-5**	**-3**	**-4**

Table 14.5-c Concentration 500 µg/plate (colonies per plate)

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
Repl. 1	117	121	12	13	113	118	226	171	11	13
Repl. 2	110	118	10	11	115	82	190	175	9	16
Repl. 3	120	117	16	13	121	134	228	180	10	17
Repl. 4	130	110	22	14	130	124	200	201	11	17
Mean	**119**	**117**	**15**	**13**	**120**	**115**	**211**	**182**	**10**	**16**
sd	**8**	**5**	**5**	**1**	**8**	**23**	**19**	**13**	**1**	**2**
f(l)	**0.96**	**1.04**	**1.00**	**0.72**	**1.12**	**1.12**	**1.27**	**0.94**	**0.63**	**0.89**
Rev. abs.	**-5**	**4**	**0**	**-5**	**13**	**12**	**45**	**-12**	**-6**	**-2**

Table 14.5-d Concentration 151 µg/plate (colonies per plate)

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Repl. 1	115	118	20	14	115	126	171	224	13	16
Repl. 2	107	120	17	13	113	131	226	222	18	13
Repl. 3	101	127	21	10	117	144	196	214	13	15
Repl. 4	113	113	15	9	120	105	198	220	9	15
Mean	**109**	**120**	**18**	**12**	**116**	**127**	**198**	**220**	**13**	**15**
sd	**6**	**6**	**3**	**2**	**3**	**16**	**22**	**4**	**4**	**1**
f(l)	**0.88**	**1.06**	**1.20**	**0.67**	**1.08**	**1.23**	**1.19**	**1.13**	**0.81**	**0.83**
Rev. abs.	**-15**	**7**	**3**	**-6**	**9**	**24**	**32**	**26**	**-3**	**-3**

Table 14.5-e Concentration 51 µg/plate (colonies per plate)

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Repl. 1	113	118	7	11	113	105	180	196	13	16
Repl. 2	114	117	9	17	115	111	228	204	8	15
Repl. 3	128	121	12	13	116	103	226	226	16	20
Repl. 4	121	120	9	19	113	116	230	214	10	17
Mean	**119**	**119**	**9**	**15**	**114**	**109**	**216**	**210**	**12**	**17**
sd	**7**	**2**	**2**	**4**	**2**	**6**	**24**	**13**	**4**	**2**
f(l)	**0.96**	**1.05**	**0.60**	**0.83**	**1.07**	**1.06**	**1.30**	**1.08**	**0.75**	**0.94**
Rev. abs.	**-5**	**6**	**-6**	**-3**	**7**	**6**	**50**	**16**	**-4**	**-1**

15 ANNEX 3: DATA OF THE SECOND EXPERIMENT

15.1 Determination of Titre

Criterion: The determination of titre should give a number of at least 10^9 cells/mL, correlating to 100 colonies/plate after dilution.

Table 15.1-a Titre Values (colonies per plate)

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Repl. 1	366	442	430	426	392	222	242	376	332	320
Repl. 2	420	424	394	422	234	382	430	354	362	318
Mean	**393**	**433**	**412**	**424**	**313**	**302**	**336**	**365**	**347**	**319**
sd	**38**	**13**	**25**	**3**	**112**	**113**	**133**	**16**	**21.2**	**1**
Assessment	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok

15.2 Sterility Control

Criterion: At the maximum, one colony per plate may grow.

Table 15.2-a Sterility (colonies per plate)

	H_2O	DMSO
Repl. 1	0	0
Repl. 2	0	0
Repl. 3	0	0
Repl. 4	0	0
Assessment	**ok**	**ok**

15.3 Spontaneous Revertants

The determined values were within the normal range of the laboratory.

15.3.1 Water

Table 15.3-a Spontaneous Revertants H_2O (colonies per plate)

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Repl. 1	118	131	10	13	97	108	206	230	19	11
Repl. 2	110	130	10	12	137	135	198	220	11	13
Repl. 3	121	121	10	11	107	138	234	210	21	15
Repl. 4	111	112	10	10	97	107	218	232	20	16
Mean	**115**	**124**	**10**	**12**	**110**	**122**	**214**	**223**	**18**	**14**
sd	**5.4**	**8.9**	**0.0**	**1.3**	**18.9**	**16.8**	**15.7**	**10.1**	**4.6**	**2.2**

15.3.2 DMSO

Table 15.3-b Spontaneous Revertants DMSO (colonies per plate)

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Repl. 1	113	149	10	22	169	109	214	252	15	15
Repl. 2	115	153	20	21	107	108	228	210	16	15
Repl. 3	105	127	17	11	94	158	210	234	15	15
Repl. 4	111	115	12	17	126	119	226	236	15	21
Mean	**111**	**136**	**15**	**18**	**124**	**124**	**220**	**233**	**15**	**17**
sd	**4.3**	**18.1**	**4.6**	**5.0**	**32.8**	**23.5**	**8.9**	**17.3**	**0.5**	**3.0**

15.4 Positive Controls

- Without metabolic activation:
  4-Nitro-1,2-phenylene diamine (NPD) in DMSO, 20 µg/plate
  Sodium azide (Na-azide) in deionised water, 1 µg/plate
- With metabolic activation:
  2-Amino anthracene (2-AA) in DMSO, 1 µg/plate
  Benzo-a-pyrene (BaP) in DMSO, 20 µg/plate

Table 15.4-a Diagnostic Mutagenes (colonies per plate)

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Substance	NPD	2-AA	NPD	BaP	Na-azide	2-AA	NPD	2-AA	Na-azide	2-AA
Repl. 1	752	544	119	138	490	560	504	532	211	100
Repl. 2	844	512	211	119	396	500	620	504	241	107
Repl. 3	460	460	181	131	332	480	684	508	257	112
Repl. 4	732	572	170	114	502	760	572	460	203	105
Mean	**697**	**522**	**170**	**126**	**430**	**575**	**595**	**501**	**228**	**106**
sd	**165**	**48**	**38**	**11**	**81**	**128**	**76**	**30**	**25**	**5**
f(I)	**6.28**	**3.84**	**11.33**	**7.00**	**3.91**	**4.64**	**2.70**	**2.15**	**12.67**	**6.24**
Rev. abs.	752	544	119	138	490	560	504	532	211	100

f(I) = increase factor, calculation see chapter „Evaluation", page 19
Rev.abs. = absolute revertants, calculation see chapter „Evaluation", page 19

15.5 Test Item [redacted]

15.5.1 Mutagenicity Test

Table 15.5-a Concentration 4999 µg/plate (colonies per plate)

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
Repl. 1	125	123	17	10	127	103	266	168	20	14
Repl. 2	127	115	10	16	120	106	214	244	13	15
Repl. 3	126	105	13	14	128	104	220	220	25	16
Repl. 4	111	116	12	11	118	105	200	210	11	17
Mean	**122**	**115**	**13**	**13**	**123**	**105**	**225**	**211**	**17**	**16**
sd	**8**	**7**	**3**	**3**	**5**	**1**	**29**	**32**	**6**	**1**
f(l)	**1.06**	**0.93**	**1.30**	**1.08**	**1.12**	**0.86**	**1.05**	**0.95**	**0.94**	**1.14**
Rev. abs.	**7**	**-9**	**3**	**1**	**13**	**-17**	**11**	**-12**	**-1**	**2**

Table 15.5-b Concentration 2528 µg/plate (colonies per plate)

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
Repl. 1	116	126	10	9	122	101	252	246	11	15
Repl. 2	105	115	10	10	120	101	210	210	13	17
Repl. 3	111	105	15	10	129	102	234	232	21	18
Repl. 4	107	117	10	10	116	100	238	222	15	21
Mean	**110**	**116**	**11**	**10**	**122**	**101**	**234**	**228**	**15**	**18**
sd	**5**	**9**	**3**	**1**	**5**	**1**	**17**	**15**	**4**	**3**
f(l)	**0.96**	**0.94**	**1.10**	**0.83**	**1.11**	**0.83**	**1.09**	**1.02**	**0.83**	**1.29**
Rev. abs.	**-5**	**-8**	**1**	**-2**	**12**	**-21**	**20**	**5**	**-3**	**4**

Table 15.5-c Concentration 1254 µg/plate (colonies per plate)

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
Repl. 1	128	116	14	13	121	110	252	266	21	17
Repl. 2	119	116	15	12	117	115	210	210	15	17
Repl. 3	120	106	16	12	120	104	208	212	18	18
Repl. 4	105	111	15	10	122	108	202	222	17	19
Mean	**118**	**112**	**15**	**12**	**120**	**109**	**218**	**228**	**18**	**18**
sd	**10**	**5**	**1**	**1**	**2**	**5**	**23**	**26**	**3**	**1**
f(l)	**1.03**	**0.90**	**1.50**	**1.00**	**1.09**	**0.89**	**1.02**	**1.02**	**1.00**	**1.29**
Rev. abs.	**3**	**-12**	**5**	**0**	**10**	**-13**	**4**	**5**	**0**	**4**

Table 15.5-d Concentration 628 µg/plate (colonies per plate)

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
Repl. 1	123	107	12	11	123	105	260	226	20	15
Repl. 2	105	118	16	17	125	106	210	232	20	16
Repl. 3	111	109	14	13	122	108	234	210	11	17
Repl. 4	113	113	11	14	121	109	210	224	13	19
Mean	**113**	**112**	**13**	**14**	**123**	**107**	**229**	**223**	**16**	**17**
sd	**7**	**5**	**2**	**3**	**2**	**2**	**24**	**9**	**5**	**2**
f(l)	**0.98**	**0.90**	**1.30**	**1.17**	**1.12**	**0.88**	**1.07**	**1.00**	**0.89**	**1.21**
Rev. abs.	**-2**	**-12**	**3**	**2**	**13**	**-15**	**15**	**0**	**-2**	**3**

Table 15.5-e Concentration 313 µg/plate (colonies per plate)

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
Repl. 1	115	105	10	14	125	109	240	226	17	10
Repl. 2	120	126	10	16	120	110	240	242	18	15
Repl. 3	105	127	10	15	126	118	240	214	20	21
Repl. 4	111	105	10	16	131	106	222	232	21	17
Mean	**113**	**116**	**10**	**15**	**126**	**111**	**236**	**229**	**19**	**16**
sd	**6**	**12**	**0**	**1**	**5**	**5**	**9**	**12**	**2**	**5**
f(l)	**0.98**	**0.94**	**1.00**	**1.25**	**1.15**	**0.91**	**1.10**	**1.03**	**1.06**	**1.14**
Rev. abs.	**-2**	**-8**	**0**	**3**	**16**	**-11**	**22**	**6**	**1**	**2**

16 ANNEX 4: DATA OF THE CYTOTOXICITY TEST

The toxicity of the following concentration was tested: 5007 µg/plate.

Per strain, four plates were incubated with the corresponding dose of the test item on maximal soft agar.

16.1 Experimental Parameters

Date of treatment	[redacted]
Concentrations tested	5007 µg/plate
Incubation time	48 hours
Incubation temperature	37 °C
Tester strains	TA97a, TA98, TA100, TA102, TA1535
Method	Plate incorporation method

16.2 Determination of Titre

Criterion: The determination of titre should give a number of at least 10^9 cells/mL, correlating to 100 colonies/plate after dilution.

Table 16.2-a Titre Values (colonies per plate)

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Repl. 1	354	430	332	432	302	420	430	302	502	334
Repl. 2	422	322	302	412	322	410	442	322	420	356
Mean	**388**	**376**	**317**	**422**	**312**	**415**	**436**	**312**	**461**	**345**
sd	**48**	**76**	**21**	**14**	**14**	**7**	**8**	**14**	**58.0**	**15.6**
Assessment	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok

16.3 Toxicity Control

The test item is considered non-toxic, if the quotient titre/tox is below 2.

Table 16.3-a 5007 µg/plate on maximal-soft-agar with culture diluted by 10^6

Strain	TA97a		TA98		TA100		TA102		TA1535	
Induction	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Repl. 1	234	282	332	246	294	232	250	272	314	230
Repl. 2	270	320	238	302	300	242	262	340	302	232
Mean	**252**	**301**	**235**	**274**	**297**	**237**	**256**	**306**	**308**	**231**
sd	**25.5**	**26.9**	**66.5**	**39.6**	**4.2**	**7.1**	**8.5**	**48.1**	**8.5**	**1.4**
Titre/Tox	**1.54**	**1.29**	**1.11**	**1.16**	**1.05**	**1.32**	**1.70**	**1.42**	**1.70**	**2.00**

17 ANNEX 5: COMPARISON WITH HISTORICAL DATA

In the following table, the history of the spontaneous revertants and positive controls of the performed experiments with these strains up to [redacted] is stated in comparison with the experiments performed within this study. Only experiments which were performed before finalisation of the study plan of this study were considered.

Table 17 -a Historical Data

Strain		TA97a		TA98		TA100		TA102		TA1535	
Induction		- S9	+ S9	- S9	+ S9	- S9	+ S9	- S9	+ S9	- S9	+ S9
H_2O	Mean	115	116	12	12	113	118	200	208	12	13
	Min	99	95	6	5	78	68	79	124	4	5
	Max	159	155	18	21	161	175	306	345	23	22
	SD	12	10	3	4	17	18	49	48	4	4
	Exp 1	124	113	15	18	107	103	166	194	16	18
	Exp 2	115	124	10	12	110	122	214	223	18	14
DMSO	Mean	116	117	12	12	114	116	201	207	12	13
	Min	89	90	6	6	89	67	95	111	5	7
	Max	167	155	19	17	155	165	313	305	21	29
	SD	12	13	3	3	14	17	50	44	3	4
	Exp 1	117	116	19	***21***	109	112	195	185	21	14
	Exp 2	111	136	15	***18***	124	124	220	233	15	17
Pos. Contr.	Mean	513	510	361	209	553	584	793	788	237	213
	Min	279	258	97	50	254	221	401	458	87	88
	Max	1139	1180	1189	849	1212	1273	1324	1345	645	873
	SD	182	206	273	161	182	190	206	206	128	153
	Exp 1	618	719	302	370	669	686	591	565	150	121
	Exp 2	697	522	170	126	430	575	595	501	228	106

The values which lie outside the range of the historical data are given in bold italics. No critical impact on the outcome of the study is expected, as the difference is marginal.

18 ANNEX 6: GENOTYPE CONFIRMATION

Date of Performance: [redacted]

18.1 Histidine Requirement

Criterion: On the biotin plates, no growth should occur, whereas on the histidine-biotin plates, the strains should grow well.
Found: Growth on histidine-biotin plates, no growth on biotin plates.
Assessment: okay

18.2 Ampicillin-(Tetracycline)-Resist. (pKM 101, pAQ1)

Criterion: After incubation for 12 hours, good growth should be observed on the ampicillin plates for all strains except TA1535. On the ampicillin-tetracycline plates, only TA102 should grow.
Found: TA97a, 98, 100, 102 grow in the presence of ampicillin, only TA102 with ampicillin and tetracycline, TA 1535 doesn't grow anywhere.
Assessment: okay

18.3 UV-Sensitivity (uvrB)

Criterion: On the irradiated side of the plates, only TA 102 should grow, whereas on the side, which was protected from light, all strains should grow.
Found: all strains grow on the non-irradiated half, only TA102 on the irradiated one.
Assessment: okay.

18.4 Crystal Violet Sensitivity (deep rough/rfa)

Criterion: The zone of inhibition has a diameter of approx. 14 mm.
Found:

Table 18.4-a Inhibition in mm

Strain	TA97a	TA98	TA100	TA102	TA1535
Repl. 1	14	14	14	14	14
Repl. 2	14	12	14	14	14
Mean	**14**	**13**	**14**	**14**	**14**

Assessment: okay.